Non-radioactive labeling of RNA transcripts *in vitro* with the hapten digoxigenin (DIG); hybridization and ELISA-based detection

Hans-Joachim Höltke and Christoph Kessler
Boehringer Mannheim GmbH, Biochemical Research Center, Nonnenwald 2, D-8122 Penzberg, FRG

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ABSTRACT

We have developed a system for the enzymatic *in vitro* synthesis of non-radioactively labeled RNA which is derivatized with the hapten digoxigenin (DIG). The labeling reaction as well as the conditions for hybridization and detection of hybrids by an antibody-conjugate and a coupled colour reaction were analyzed and adapted for high sensitivity and low background. In addition, data on the performance and sensitivity of digoxigenin-labeled RNA probes in Southern and Northern blots are presented.

INTRODUCTION

RNA probes are commonly synthesized with the RNA polymerases from bacteriophages SP6, T7 and T3 by *in vitro* transcription of DNA, cloned into appropriate transcription vectors (1−7). The advantages of RNA probes, e.g. single strandedness, no self-complementarity, defined length and higher stability of RNA/DNA-hybrids have been widely exploited in different hybridization applications (2, 3, 8, 9). In most cases radioactively labeled probes were used so far with this technique; however, these probes have the disadvantage of instability, low resolution, and all the pitfalls of handling radioisotopes.

Therefore a number of methods have been developed for the non-radioactive labeling and detection of nucleic acids (10). Most of them involve the modification of the nucleic acid probe with biotin (11) and the detection of hybrids by streptavidin or avidin (11−13) coupled to reporter molecules, e.g. fluorescent compounds (14) or enzymes (11, 15), which themselves can be visualized by catalysis of a colour (13), luminescence (16), fluorescence (17) or coupled signal amplification (17) reaction.

Biotin was used for non-radioactive labeling of RNA either by direct incorporation of biotin-coupled ribonucleoside-triphosphate in the *in vitro* transcription reaction (18), or by incorporation of allylamino-UTP and subsequent coupling of biotin-N-hydroxysuccinimide to the allylamino group (11). Both methods did not receive wide acceptance, because substitution of e.g. UTP by biotin-UTP inhibits quite strongly the different phage-coded RNA polymerases (18); this results in a low yield of labeled RNA. Allylamino-UTP on the other hand is a quite efficient substrate for the RNA polymerases, but the subsequent chemical coupling to the succinimide ester is not as efficient as direct enzymatic incorporation (1).

In addition, biotin-labeled nucleic acid probes often give rise to unspecific side reactions, especially when used for *in situ* hybridizations, as biotin itself occurs in almost all natural materials (19). Furthermore, streptavidin or avidin used for detection of the hybrids tend to bind unspecifically to tissues and membranes (especially highly charged nylon membranes) resulting in increased unspecific background signals (20).

In order to avoid the drawbacks of the biotin/(strept)avidin system we have developed an alternative method for labeling nucleic acid probes with the cardenolide digoxigenin (DIG) which occurs only in *Digitalis* plants (21−25). For detection of digoxigenin-labeled hybrids Fab-fragments of an highly specific polyclonal sheep antibody coupled to alkaline phosphatase are applied. In this publication we report on the synthesis and application of digoxigenin-labeled RNA probes in various blot formats. The most important parameters influencing synthesis, hybridization and detection of digoxigenin-modified RNA probes will be described.

MATERIALS AND METHODS

Materials

1. Reagents

ATP, GTP, CTP and UTP; restriction endonucleases *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Hin*fI, *Pvu*II; SP6, T7 and T3 RNA polymerases; DNAseI, RNAse-free; RNAse inhibitor; alkaline phosphatase from calf intestine, enzyme label for enzyme immunoassay (AP); <DIG>AP (polyclonal sheep anti-digoxigenin-antibody(Fab):alkaline phosphatase-conjugate [750 units/ml]); blocking reagent (casein from non-fat dry milk obtained by acid precipitation [acetic acid, pH 4.6] and subsequent solubilization [NaOH, 0.1 M] of milk proteins); BCIP (5-bromo-4-chloro-3-indolylphosphate); NBT (nitroblue tetrazolium salt); plasmids pBR328, pSPT18, pSPT19, pSPT18-*neo*, pSPT19-*neo*, pT3T7*lac*; DNA from herring sperm; SP6/T7 transcription kit; digoxigenin-ELISA test kit; spermidine, DTT (dithiothreitol), Tris (tris[hydroxymethyl]-aminomethane) and SDS (sodium dodecyl sulfate) were from Boehringer Mannheim.

Human placenta DNA was from Clontech. [α-^{32}P]-cytosine-5'-triphosphate, triethylammonium salt ([α-^{32}P]CTP; ca. 400 Ci/mmol) was from Amersham Buchler. Phenol, chloroform, ethanol (99%, p.a.), formamide, formaldehyde, LiCl, EDTA

(ethylene[dinitrilo]-tetraacetic acid), TCA (trichloroacetic acid), NaCl, $MgCl_2$ and Na-citrate were from Merck. Maleic acid, Na_2-salt was from Fluka. N-lauroyl-sarkosine and diethylpyrocarbonate (DEPC) were from Sigma. X-OMAT S X-ray films and intensifier screens were from Kodak. For routine tests nitrocellulose BA85 membrane was from Schleicher and Schuell; nylon membranes were from Pall (Biodyne B) and from Amersham (Hybond N); the suppliers of membranes used for systematic comparisons are given below.

2. Solutions

DEPC-treated water: bidest H_2O was stirred with 0.1% (v/v) diethylpyrocarbonate for 30 min at 25°C and autoclaved. *20×SSC*: NaCl, 3 mol/l; Na-citrate, 0.3 mol/l; pH 7.0/25°C. *DNA dilution buffer*: 50 μg/ml herring sperm DNA in Tris-HCl, 10 mmol/l; EDTA, 1 mmol/l; pH 8.0/25°C. *RNA dilution buffer*: formaldehyde, 20×SSC and DEPC-treated water were mixed in a ratio of 2:3:5. *10×concentrated transcription buffer*: Tris-HCl, 0.4 mol/l, pH 8.0/25°C; $MgCl_2$, 60 mmol/l; dithiothreitol, 0.1 mol/l; spermidine, 20 mmol/l; NaCl, 0.1 mol/l; RNAse inhibitor, 1 U/μl. *10×concentrated DIG-NTP labeling mixutre*: ATP, 10 mmol/l; GTP, 10 mmol/l; CTP, 10 mmol/l; UTP, 6.5 mmol/l; DIG-UTP, 3.5 mmol/l; pH 7.5/25°C. *BCIP solution*: 50 mg/ml 5-bromo-4-chloro-3-indolylphosphate, toluidinium salt in dimethylformamide, 100% (v/v). *NBT solution*: 75 mg/ml nitroblue tetrazolium salt in dimethylformamide, 70% (v/v). *Hybridization solution*: formamide, 50% (v/v); 5×SSC; blocking reagent, 5% (w/v); N-lauroyl-sarkosine, 0.1% (w/v); SDS, 0.02% (w/v); pH 7.0/25°C. *Blocking buffer*: Blocking reagent, 1% (w/v) in buffer 1. *Buffer 1*: Tris-HCl, 100 mmol/l; NaCl, 150 mmol/l; pH 7.5/25°C. *Buffer 2*: Tris-HCl, 100 mmol/l; NaCl, 100 mmol/l; $MgCl_2$, 50 mmol/l; pH 9.5/25°C. *Buffer 3*: Tris-HCl, 10 mmol/l; EDTA, 1 mmol/l; pH 8.0/25°C. *AP substrate solution, freshly prepared*: 45 μl NBT solution (90 mmol/l) and 35 μl BCIP solution (120 mmol/l) were added to 10 ml buffer 3.

Methods

DNA and RNA isolation and electrophoresis, Southern-, Northern- and dot-blots: Standard protocols were followed as described in (26, 27). For the synthesis of digoxigenin-labeled *neo*-specific RNA probes the *neo* gene (28) was cloned into pSPT18 and pSPT19 (29); after linearization with either *Eco*RI or *Pvu*II both sense and antisense labeled RNA could be generated in the presence of digoxigenin-labeled UTP (DIG-UTP) as run-off transcripts with SP6 and T7 RNA polymerase, respectively. β-actin RNA probes were obtained by cloning a 544 bp *Sal*I/*Xho*II-fragment of the human β-actin cDNA clone pHFβA-1 (30) into the *Sal*I/*Bam*HI-sites of pT3T7*lac* (29) and subsequent *in vitro* transcription (Müller,J., unpublished results).

RNA labeling with digoxigenin: DIG-UTP (digoxigenin-O-methylcarbonyl-ε-aminocaproyl-[5-(3-aminoallyl)-2'-uridine-5'-triphosphate], Na_4) was synthesized analogous to (24). The digoxigenin-modified nucleotide was prepared by reaction of 3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester with 5-aminoallyl-substituted UTP. The former compound was obtained by derivatization of the digoxigenin aglycon via a five-step procedure by 1. coupling with diazo acetic acid ethyl ester, 2. saponification with $KHCO_3$, 3. condensation with N-hydroxysuccinimide and dicyclocarbodiimide, 4. coupling with ε-aminocaproic acid, and 5. additional condensation with N-hydroxysuccinimide and dicyclocarbodiimide.

5-aminoallyl-UTP had been synthesized analogous to 5-aminoallyl-dUTP as described in (24) but starting from uridine-5'-triphosphate instead of deoxyuridine-5'-triphosphate (K. Mühlegger, unpublished results).

For the labeling reaction the following components were added to a microfuge tube on ice: 1 μg of (linearized) template DNA, 2 μl 10×concentrated DIG-NTP labeling mixture, 2 μl 10×concentrated transcription buffer; then the reaction volume was made up to 18 μl with sterile redistilled H_2O, and finally 2 μl (40 U) SP6, T7 or T3 RNA polymerase were added. Some reactions contained in addition 1 μl (20 U) of RNAse inhibitor. Incubation was performed for 2 hrs at 37°C. Occasionally the DNA template was digested by adding 2 μl (20 U) DNAse, RNAse-free and incubation for a further 15 min at 37°C. The reaction was stopped by adding 2 μl EDTA, 0.2 mol/l; pH 8.0/25°C. The labeled RNA was precipitated with 2.4 μl LiCl, 4 mol/l, and 75 μl prechilled (−20°C) ethanol and mixing. The precipitate was left for at least 30 min at −70°C or 2 hrs at −20°C. The precipitate was centrifuged at 12,000 g, washed with cold ethanol (70% [v/v]), dried under vacuum and dissolved in 100 μl DEPC-pretreated water. For determining the amount of synthesized digoxigenin-labeled RNA, [α-^{32}]CTP was added as a tracer and its incorporation measured by trichloroacetic acid (TCA) precipitation. Radioactive RNA probes were synthesized using the SP6/T7 transcription kit following exactly the recommended procedure given by the supplier.

Hybridization with digoxigenin-labeled RNA probes: Nitrocellulose was presoaked in water and 20×SSC. Nylon membranes were used without pretreatment. DNA or RNA was bound to nitrocellulose by baking for 2 hrs in vacuum at 80°C and to nylon membranes either by baking for 2 hrs or by UV crosslinking at λ = 302 nm with a transillumination device for 3 min (31). Membranes were prehybridized in a sealed plastic bag or box with at least 20 ml hybridization solution per 100 cm^2 of filter for at least 1 h at 55°C (DNA) or at 68°C (RNA). The solution was shaken several times. For probe-hybridization the solution was replaced by hybridization solution containing digoxigenin-labeled RNA; 50−200 ng of digoxigenin-labeled RNA per ml hybridization solution were used. A volume of 2.5 ml of hybridization solution was applied per 100 cm^2 of filter, for very small filters slightly more. Hybridization was performed for 16 hrs at 55°C (DNA) or at 68°C (RNA); the solution was re-distributed occasionally. Hybridization solutions containing digoxigenin-labeled RNA were stored at −20°C. They were freshly denatured and re-used up to four times with the same sensitivity and low background as in the first experiment. Hybridizations with radioactive RNA probes were performed under identical conditions using 5×10^6 cpm/ml. The membranes were washed for 2×5 min at room temperature with at least 50 ml of 2×SSC, 0.1% (w/v) SDS per 100 cm^2 and 2×15 min at 68°C with 0.1×SSC; 0.1% (w/v) SDS. Membranes were then used directly for detection of hybridized RNA or were stored air dried for later detection.

Immunological detection of digoxigenin-labeled RNA: All of the following incubations were performed at room temperature and except for the colour reaction with shaking or mixing. The cited volumes of the solutions refer to a membrane-size of 100 cm^2 and were adjusted in the various experiments according to the

membrane sizes used. The membranes were washed briefly (1 min) with 100 ml buffer 1. The membranes were then incubated for 30 min in 100 ml blocking solution. <DIG>AP was freshly diluted to 150 mU/ml (1:5000) in blocking solution (dilute antibody-conjugate solutions were stable only for approximately 12 hrs at 4°C). After discarding blocking solution, the membranes were incubated for 30 min with 20 ml of diluted <DIG>AP solution. The unbound <DIG>AP was removed by washing 2×15 min with 100 ml of buffer 1. Finally the membranes were equilibrated for 2 min with 20 ml of buffer 2 and then incubated with 10 ml freshly prepared AP substrate solution sealed in a plastic bag or in a suitable box in the dark. The coloured precipitate started to form within a few minutes. When the desired spots or bands were prominent enough, the reaction was stopped by washing the membrane for 5 min with 50 ml of buffer 3; the reaction was usually complete after 24 hrs. The results were documented by photocopying or photography of the wet membrane. The membrane was dried at room temperature or by baking at 80°C and stored at room temperature in the dark; colours faded upon drying. The colour could be re-vitalized by wetting the membrane with buffer 3.

Synthesis of the digoxigenin polyclonal antibody:alkaline phosphatase conjugate: The conjugates between polyclonal sheep digoxigenin-specific antibody Fab-fragments and alkaline phosphatase from calf intestine (<DIG>:AP) were obtained as follows: Polyclonal antibodies against digoxigenin were raised in sheep by injecting digoxigenin coupled to bovine serum albumin or edestin (32, 33). The IgG-fraction was cleaved with papain, the Fab-fragments were separated from Fc-fragments by DEAE-chromatography and subsequently immunopurified by affinity chromatography on digoxigenin-aminospherosil adsorbant. Thiol-reactive maleimide groups were introduced by derivatization of the Fab-fragments with maleimidohexanoyl-N-hydroxysuccinimide ester (MHS). Alkaline phosphatase was activated by S-acetylmercaptosuccinic anhydride (SAMSA) and the blocked SH-groups deacetylated with hydroxylamine (34). Alkaline phosphatase (AP) was coupled to a molar excess of Fab-fragments and the raw conjugate was size-fractionated on Sephacryl S300. The fractions in the appropriate high molecular weight size range were selected by testing for high enzymatic activity (35) and for optimal function in non-radioactive DNA detection.

RESULTS

Conditions for efficient digoxigenin incorporation

For most of the following optimizations the standard assay format described above was used. Figure 1 shows the principle of the digoxigenin incorporation during *in vitro* transcription. For example, for digoxigenin-labeling of *neo* RNA transcripts the *neo* gene (28) was cloned into pSPT18 and pSPT19 transcription vectors; after linearization with either *Eco*RI or *Pvu*II both sense and antisense RNA could be transcribed in the presence of digoxigenin-labeled UTP (DIG-UTP) as run-off transcript with SP6 or T7 RNA polymerases, respectively. The structure of DIG-UTP is shown in Figure 2. It should be noted that the digoxigenin-hapten is connected with the nucleotide moiety by an alkaline-stable spacer; this allows alkaline treatment which might be of advantage particularly for *in situ* hybridizations (36, 37). Transcription products were analyzed according to: 1) high yield, as calculated from incorporation of the radioactive tracer

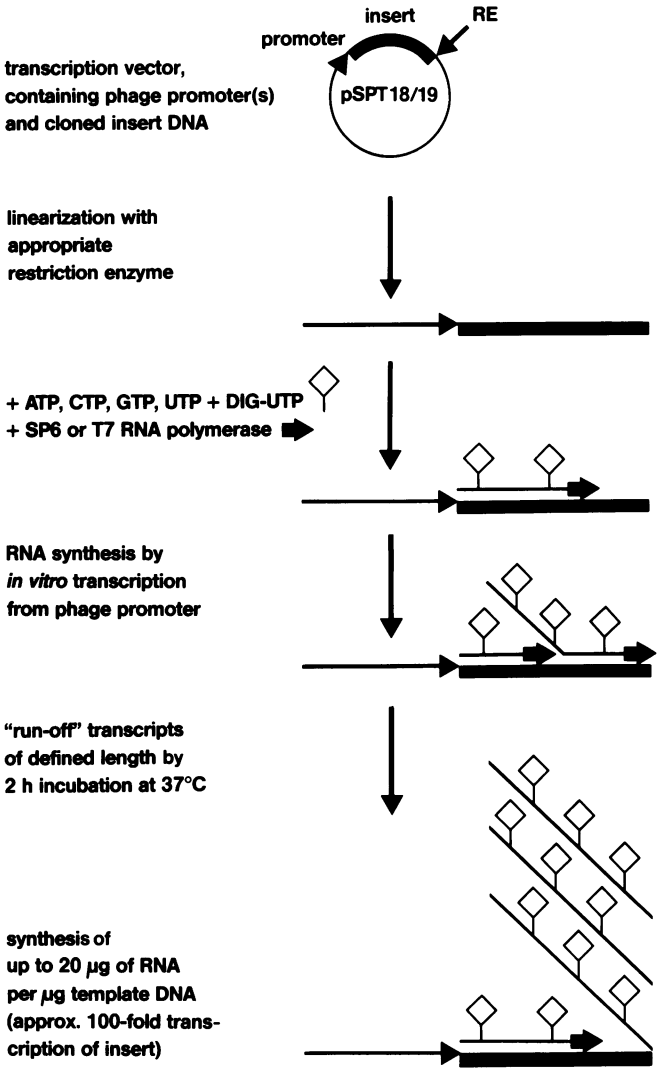

Figure 1. Reaction scheme of labeling RNA with digoxigenin by *in vitro* run-off transcription in the presence of DIG-UTP.

[α-^{32}]CTP and intensity of EtBr-stained transcript after agarose gel electrophoresis; 2) full length of transcripts as analyzed by agarose gel electrophoresis; 3) sensitivity and background in hybridization reactions. Both RNA:DNA and RNA:RNA hybridizations with digoxigenin-labeled *neo* RNA were tested to either pSPT18/19-*neo* DNA or to unlabeled *neo* RNA transcripts.

Different buffer compositions were tested under otherwise constant standard conditions (1 µg template, DIG-NTP labeling mixture, 40 U RNA polymerase). A buffer similar to that published by Nielson and Shapiro (5) containing a higher concentration of DTT and additionally NaCl and RNAsin as compared to the original conditions of Melton *et al.* (2) gave the highest yields of full length digoxigenin-labeled transcripts. Maximum yields were obtained after 2 hrs incubation; longer incubation periods resulted in decreased yields (Figure 3). Including PEG 8000 at 10 to 20% (w/v) (38) increased the yield of transcripts; however, these results were not fully reproducible and analysis on agarose gels showed a smear beside the expected transcript band.

Using 1 µg of template DNA and increasing the concentration of NTPs from 0.5 mM to 1 mM increased the yield of

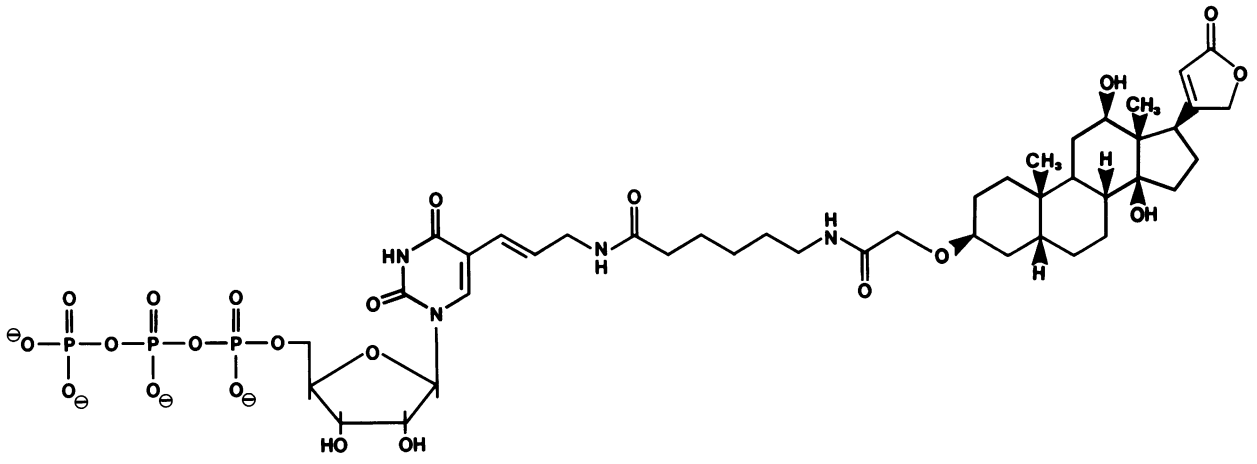

Figure 2. Structure of DIG-UTP. The compound shown with a spacer length of 9 atoms between UTP and digoxigenin exhibited highest sensitivity as compared to compounds with shorter spacers.

digoxigenin-labeled transcripts from 6.5 to 10 μg; higher concentrations of NTPs decreased the yield, with NTPs at 2 mM only 8 μg transcripts were synthesized. For this reason a concentration of 1 mM of each NTP was chosen as standard condition. Although increasing the amount of RNA polymerases from 1 U/μl to 2 U/μl of assay volume almost led to a doubling of the yield from 6 μg to 10 μg transcripts per μg template DNA, an even higher concentration of enzyme up to 5 U/μl final enzyme concentration resulted in only a slight additional increase in yield.

Removing the template DNA after the transcription reaction by DNAse-treatment did not have any significant effect on both sensitivity of hybridization with digoxigenin-labeled RNA or background. Phenol extraction of transcription assays resulted in poor recovery of the digoxigenin-labeled RNA. This was attributed to partitioning of the modified RNA into the organic phase. Digoxigenin-labeled RNA was purified from unincorporated nucleotides by ethanol precipitation. Addition of a carrier like tRNA or glycogen proved unnecessary, as no loss of DIG-RNA by ethanol precipitation was observed. When transcripts were used in hybridization reactions without prior purification from unincorporated nucleotides by ethanol precipitation a slight increase in background reaction was observed.

For the synthesis of even larger amounts of digoxigenin-labeled RNA the volume of the standard transcription assay was linearly scaled up to 100 fold. With 0.1 mg linear template DNA, 0.2 ml 10×DIG-NTP labeling mixture, 0.2 ml 10×transcription buffer and 4 kU T7 RNA polymerase in a final volume of 2 ml; 1.3 mg of digoxigenin-labeled RNA transcripts could be synthesized within 2 hours.

Ratio of DIG-UTP to unlabeled NTPs during labeling reaction

To determine the effect of labeling density on the yield of digoxigenin-modified transcripts as well as sensitivity obtained with the digoxigenin-modified RNA probe in hybridization assays, the ratio of DIG-UTP to unmodified UTP in the labeling reaction was increased stepwise from 0 to 100%. The total concentration of DIG-UTP:UTP in the assay was kept constant at 1 mM as for each of the other NTPs (ATP, CTP, GTP). The data in Table 1 show, that an increase in the ratio of DIG-UTP to UTP caused the rate of RNA synthesis to decrease. Without DIG-UTP in the transcription assay approximately 20 μg

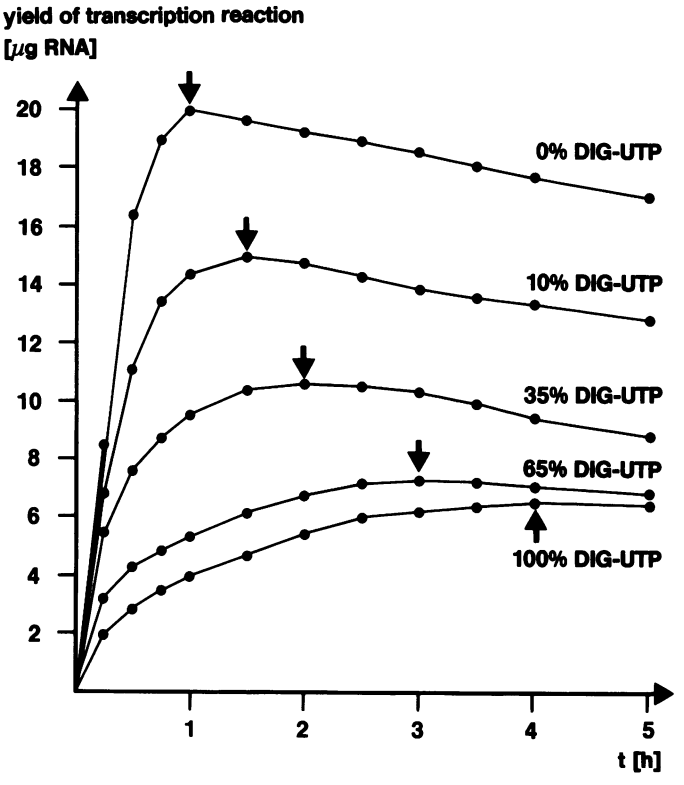

Figure 3. Dependence of kinetics and yield of synthesized digoxigenin-labeled RNA on the ratio of DIG-UTP:UTP during the *in vitro* transcription reaction. 1 μg of linear pSPT18-*neo* DNA was used as template for standard transcription reactions (37°C, 20 μl final volume per reaction). The concentration of each NTP was kept constant at 1 mmol/l; only the ratio of DIG-UTP:UTP was varied.

transcripts were synthesized in 1 h. Substitution of DIG-UTP completely for unlabeled UTP reduced both the rate of transcription and the yield significantly; after 4 hours only 6.5 μg of RNA were synthesized. As measured independently by DIG-ELISA, DIG-UTP was incorporated approximately 30–40% as efficiently as unlabeled UTP. This value is in agreement with the value of 0.33 for the ratio of synthesized RNA at 100 and 0% DIG-UTP (Table 1).

The RNAs with different hapten densities were used for

Table 1. Dependence of the yield of digoxigenin-labeled RNA on the ratio of DIG-UTP:UTP in the transcription reaction. 1 μg of linear pSPT18-*neo* DNA was used as template for standard transcription reactions (2 hrs, 37°C, 20 μl final volume per reaction). The concentration of each NTP was kept constant at 1 mmol/l; only the ratio of DIG-UTP:UTP was varied. The yields were determined at the respective optima which are marked by an arrow in Fig. 3. Sensitivity was measured in homologous RNA:RNA and RNA:DNA hybridizations at optimal signal to noise ratios. Background on highly charged nylon membranes was defined as follows: −, no background; +, low background; ++, high background; +++, very high background.

ratio DIG-UTP:UTP (%)	0	5	10	15	20	25	35	50	65	75	100
yield of DIG-RNA (μg)	20	17	15	13	12	11	10	8	7.5	7	6.5
sensitivity (pg)	−	30	3	0.3	0.1	0.1	0.03	0.03	0.1	0.3	1
background	−	−	−	−	−	−	−	+	+	++	+++

homologous hybridizations; in each case the concentration of labeled RNA was adjusted to obtain the highest relative sensitivity. RNA labeled with a ratio of DIG-UTP to UTP of 1:2 to 1:3 resulted in highest sensitivity in hybridizations to homologous DNA or RNA (Table 1). The ratio of 1:2 to 1:3 between DIG-UTP and UTP represented an optimum; both alterations to higher and lower ratios resulted in a decreased sensitivity. In addition, with higher ratios the background was enhanced.

RNA labeled with the optimal 10×DIG-NTP labeling mixture, containing ATP, GTP, CTP, 10 mmol/l each, UTP, 6.5 mmol/l, and DIG-UTP, 3.5 mmol/l, resulted in RNA transcripts which are statistically labeled at every 25th nucleotide with digoxigenin. This relatively high labeling density obtained by enzymatic incorporation resulted in a slightly reduced mobility during agarose gel-electrophoresis (data not shown); in contrast, labeling of RNA by photodigoxigenin results in a tenfold reduced labeling density (every 200−400 nucleotides) without significant alteration of the migration mobility of the digoxigenin-modified RNA during gel-electrophoresis (25).

DIG-labeled RNA-probes were stored for more than 2 years; there was no degradation of the RNA and the sensitivity in hybridization was unchanged compared to newly synthesized probes.

Types of membrane

For dot-blots and Southern-blots nitrocellulose and different brands of nylon membranes were evaluated (e.g. nitrocellulose BA85 and Nytran 13, Schleicher and Schuell; Hybond C, Hybond N and Hybond N+, Amersham; Biodyne A and Biodyne B, Pall; Biotrace RP and Biotrace NT, Gelman; Genescreen Plus, NEN; Zetabind, Cuno; Zeta-Probe, Bio-Rad; Nitroplus 2000 and Magnagraph, MSI). Background and sensitivity varied between different brands of nylon-based membranes, but also between different lots of the same brand. In general, there was less background colouring with nitrocellulose than with nylon membranes; background on nylon membranes was correlated with the binding capacity of the membranes. Those with high binding capacity (like Pall Biodyne B) exhibited higher sensitivity, but also increased background. The modification of the blocking buffer described below reduced the background markedly. Fixation of sample DNA or RNA by UV-crosslinking (Stratalinker, Stratagene) gave superior results (i.e. stronger signals and better sensitivity) than fixation by baking the membranes at 80°C; this effect was most significant with amphoteric nylon membranes (Biodyne A, Pall; Hybond N, Amersham).

Hybridization with digoxigenin-labeled RNA probes

Hybridization buffers of different compositions were tested for specific hybrid formation with digoxigenin-labeled RNA probes.

Table 2. Dependence of the sensitivity of detection of digoxigenin-labeled RNA on the concentration of digoxigenin-labeled RNA in the hybridization step and on the duration of the colour reaction. Dot-blots of pSPT18-*neo* DNA or *neo* RNA were hybridized with different concentrations of digoxigenin-labeled *neo* RNA. The given concentrations refer to *labeled* RNA calculated on the basis of a yield of 10 μg total RNA per μg template DNA in the standard transcription assay. The sensitivity was determined after 1, 3 or 16 hours of colour development.

Concentration of labeled RNA	sensitivity after colour development for 1 h	3 hrs	16 hrs
2 ng/ml	>10 pg	10 pg	1 pg
5 ng/ml	10 pg	3 pg	1 pg
10 ng/ml	3 pg	1 pg	0.3 pg
20 ng/ml	3 pg	1 pg	0.3 pg
50 ng/ml	1 pg	0.3 pg	0.1 pg
100 ng/ml	1 pg	0.3 pg	0.03 pg
200 ng/ml	1 pg	0.3 pg	0.03 pg
500 ng/ml	1 pg	0.1 pg	backgr.

Only buffers containing 50% (v/v) formamide gave satisfactory sensitivity and low background. The addition of SDS to 0.02% (w/v) and N-lauroyl-sarkosine to 0.1% (w/v) increased sensitivity and reduced background. 5×SSC was chosen as salt and buffer, even though SSPE or $NaPO_4$ gave equivalent results. The addition of tRNA did not influence sensitivity and background; dextran sulphate led to increased background and did not improve sensitivity. Essential for reduction of background was the addition of blocking reagent up to 5% (w/v); blocking reagent could be replaced by 5×Denhardt's, but background was lower with the blocking reagent (data not shown). It was important to keep the filters wet between pre- and probe-hybridization to avoid background.

Different concentrations of digoxigenin-labeled RNA were used for probe-hybridizations to homologous DNA or RNA. The sensitivity obtained for different periods of the colour reaction was determined for the various probe concentrations (Table 2). 10 ng of labeled RNA per ml hybridization solution were sufficient to detect 0.3 pg homologous DNA or RNA after 16 hrs of colour reaction. Higher concentrations of labeled RNA (>50 ng/ml) resulted in faster detection (3 hrs) and higher sensitivity (0.03 pg). Increasing the concentration to more than 200 ng/ml did not increase sensitivity, but resulted in higher background.

For analysis of the optimal temperature for pre- and probe-hybridizations a series of dot-blots between digoxigenin-labeled *neo* RNA as probe and homologous target DNA (pSPT18-*neo* DNA) or RNA (*neo* RNA) as well as heterologous DNA (lambda DNA, placenta DNA) or RNA (yeast ribosomal RNA) as controls were performed at 37, 42, 45, 50, 55, 60, 68 and 75°C in standard hybridization buffer containing 50% (v/v) formamide. Highest sensitivity correlated with the absence of unspecific

hybridization signals was observed for DNA dots between 45 and 60°C and for RNA dots between 55 and 68°C. These results were confirmed with different RNA probes and in Southern- as well as Northern-hybridizations.

Detection of digoxigenin-labeled RNA probe

The presence of 1% (w/v) blocking reagent effectively blocked the membrane against unspecific binding of antibody conjugate. The blocking reagent gave less background colouring than BSA, even when BSA was used at higher concentrations (3% [w/v]) or at elevated temperatures (65°C). Blocking reagent reduced background to a greater extent than skim dry milk. For the direct detection of spots of digoxigenin-labeled RNA on membranes it was necessary to destroy RNAse activity in the blocking buffer. Sterilizing by autoclaving was not possible as precipitates formed and a strong background colouring was observed. Treatment of the blocking buffer with diethylpyrocarbonate (DEPC) for 30 min at room temperature followed by heating to 60°C for 2 hours allowed sensitive detection of spotted labeled RNA, but led to increased background, especially on nylon membranes. Therefore alternative means to sterilize blocking buffer were tested; best results were obtained with a modified blocking buffer. Blocking reagent was dissolved to 5% (w/v) in a solution of maleic acid, Na_2-salt, 0.1 mol/l, treated with DEPC for 30 min at 25°C and then sterilized by autoclaving. For use as blocking buffer, this concentrated solution was diluted 5-fold to a final concentration of blocking reagent of 1% (w/v) with a sterile solution of maleic acid, Na_2-salt, 0.1 mol/l.

For detection of digoxigenin-labeled RNA:DNA or RNA:RNA hybrids in different blot formats sterilization of the blocking buffer was not necessary and both the Tris-buffered and maleic acid containing blocking buffers with 1% blocking reagent gave equivalent results (data not shown).

The sheep polyclonal antibodies used showed 100% cross-reactivity with digoxigenin and digoxin, but <1% cross-reactivity with other steroids such as human estrogens (e.g., estradiol) or androgens (e.g., testosterone) (21). <DIG>-AP-conjugates were size-fractionated by chromatography on Sephacryl S300. Highest sensitivity and lowest background were obtained with conjugates of MW >750 kD. For conjugation the Fab-fragments were used because in this case the background was markedly reduced as compared to conjugates containing complete antibodies. The concentration of <DIG>AP-conjugates in the detection reaction was varied over a wide range (50−1000 mU/ml) with almost identical sensitivities and background signals. For the standard reaction a medium concentration of 150 mU/ml was chosen. Diluting the antibody-conjugate in buffer 2 instead if buffer 1 as described earlier (21) reduced background additionally.

5-Bromo-4-chloro-3-indolylphosphate (BCIP) was used in combination with a variety of tetrazolium salts (e.g., thiazolyl tetrazolium bromide, neotetrazolium chloride, tetranitroblue tetrazolium chloride, tetrazoliumblue chloride, jodo-nitro tetrazolium chloride) for the detection of <DIG>AP bound to digoxigenin-labeled RNA. Because NBT gave the most visible coloured precipitate (dark purple-blue), this tetrazolium salt was chosen as standard substrate. The other evaluated tetrazolium salts (21) may be useful, when different colours are desired for special applications. The colours appeared differently on nitrocellulose or nylon membranes. Increasing the concentration in the colour reaction of BCIP and NBT to >0.4 mmol/l each did not result in higher sensitivity or in faster colour reaction. Therefore, concentrations of 0.4 mmol/l were used in the standard reaction.

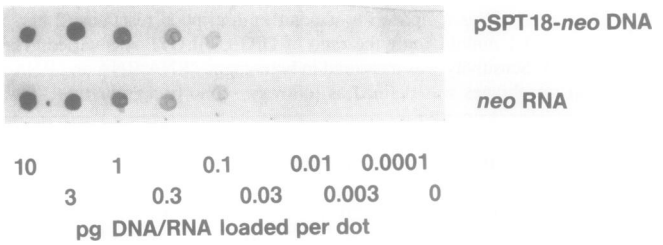

Figure 4. Sensitivity of digoxigenin-labeled RNA probes in dot-blot hybridizations. pSPT18 *neo* DNA was diluted with DNA dilution buffer containing herring sperm DNA, heat denatured and spotted onto nylon membranes (Pall Biodyne B). *Neo* sense-RNA was diluted in RNA dilution buffer, denatured and also spotted onto the nylon membranes. Hybridization to both spotted DNA and RNA was with 100 ng/ml digoxigenin-labeled *neo* antisense-RNA for 16 hrs; hybrids were detected according to the standard protocol, after 16 hours of colour development.

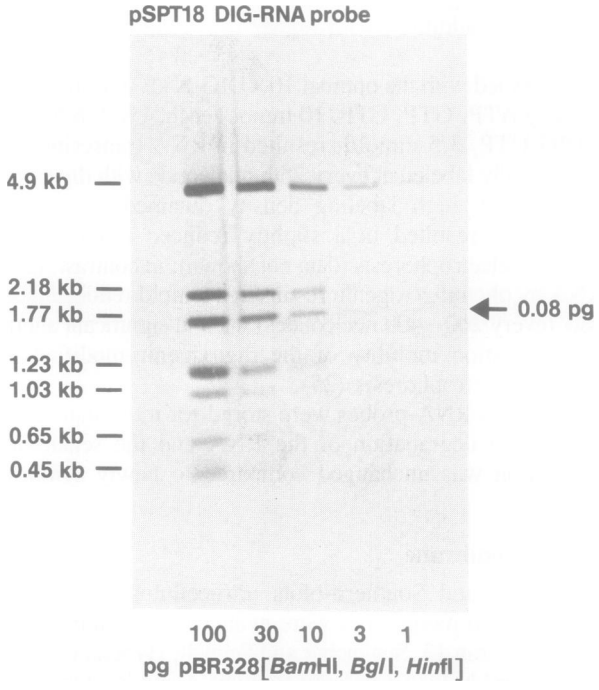

Figure 5. Sensitivity of digoxigenin-labeled RNA probes in Southern-blot hybridizations of plasmid sequences. Decreasing amounts of pBR328 DNA, which were digested separately with *Bam*HI, *Bgl*I and *Hin*fI and mixed in a ratio of 2:3:3, were Southern-blotted to a Pall Biodyne B membrane and hybridized with 100 ng/ml digoxigenin-labeled pSPT18-vector transcripts; pSPT18 is partially homologous to pBR328. The arrow marks a fragment containing 0.08 pg homologous DNA which can be detected after 16 hours of colour development.

Application of digoxigenin-labeled RNA probes

Digoxigenin-labeled RNA probes were applied in various hybridization formats (26, 27) including dot-, Southern-, Northern- and genomic blots.

For evaluation of the sensitivity of digoxigenin-labeled RNA probes in dot-blots digoxigenin-labeled *neo* RNA was hybridized to both pSPT18-*neo* DNA or *neo* RNA transcripts (28). As shown

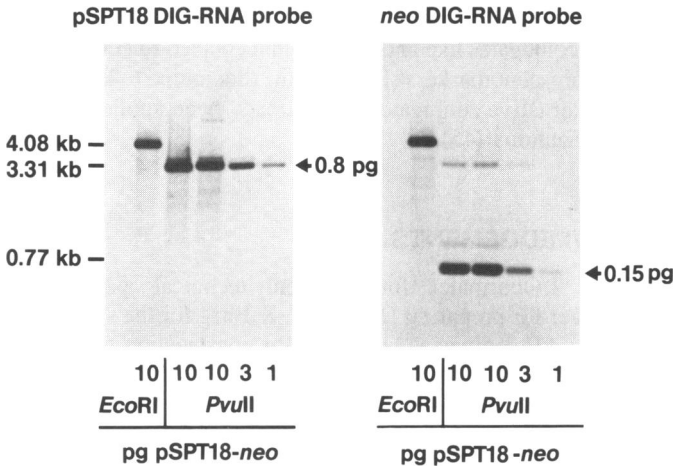

Figure 6. Sensitivity of digoxigenin-labeled RNA probes in Southern-blot hybridizations of cloned *neo* gene sequences. pSPT8-*neo* was either digested by *Eco*RI or by *Pvu*II. *Eco*RI linearizes the plasmid, so that the same band was detected by the pSPT18 vector-probe and the *neo* RNA probe. A fragment of 0.77 kb containing only *neo*-sequences was released upon *Pvu*II-digestion. Therefore different bands were visualized after hybridization and colour reaction for 16 hours to either the vector or the *neo*-RNA probes.

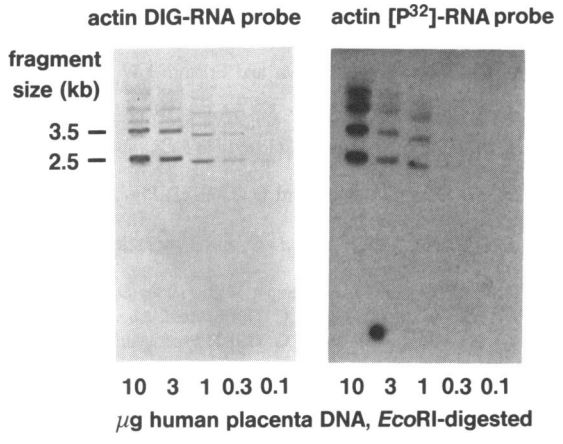

Figure 7. Comparison of non-radioactive and radioactive detection of human β-actin genes in genomic blots. (left) For non-radioactive detection of β-actin genes decreasing amounts (10−0.1 μg) of human placenta DNA were digested with *Eco*RI, Southern-blotted to a nylon membrane (Hybond N) and hybridized with 100 ng/ml digoxigenin-labeled β-actin RNA transcripts; the colour development during detection was for 16 hours. The fragment sizes were determined by comparison with lambda-*Hin*dIII/*Eco*RI-fragments as DNA-markers. (right) For radioactive detection of β-actin genes an analogous blot was hybridized with 5×10^6 cpm/ml [^{32}P]-labeled β-actin RNA transcripts; the exposure of the X-Ray film was for 16 hours.

in Figure 4 as low as 0.1−0.03 pg homologous DNA or RNA were detected after 16 hours colour reaction.

A sensitivity in the sub-picogram range was also observed in Southern-blots. A mixture of pBR328 fragments obtained by cleavage with *Bam*HI, *Bgl*I and *Hin*fI, were separated by electrophoresis and blotted onto a nylon membrane, hybridization was performed with digoxigenin-labeled RNA transcripts of pSPT18 vector DNA. In this blot a fragment containing 0.08 pg homologous DNA was detected (Figure 5). In an alternative experiment *Eco*RI and *Pvu*II digests of pSPT18-*neo* were

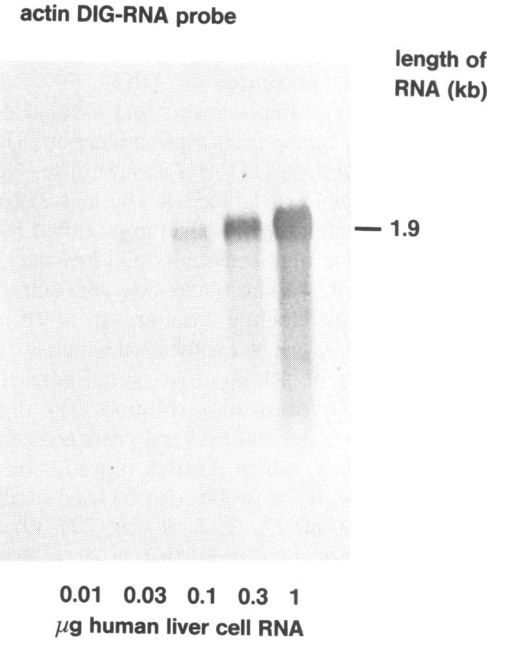

Figure 8. Detection of mature and polyadenylated β-actin mRNA in total liver cell RNA. Total liver cell RNA was separated in a denaturing formaldehyde agarose gel and transferred to a Pall Biodyne B membrane. Hybridization was with 100 ng/ml of digoxigenin-labeled β-actin antisense-RNA transcripts; the colour development was for 16 hours.

hybridized to either digoxigenin-labeled pSPT18 vector-transcripts or *neo*-specific run-off transcripts (Figure 6). The fragments which are still visible in the respective lanes loaded with 1 pg total DNA correspond to 0.8 and 0.15 pg homologous DNA. These examples also demonstrate the high sensitivity, resolution and low background obtained with digoxigenin-labeled RNA probes.

To evaluate the sensitivity of digoxigenin-labeled RNAs in genomic blots, labeled RNA probes specific for human β-actin genes (30) were applied for hybridization to human placenta DNA. In this experiment, the digoxigenin-labeled β-actin-specific RNA probe was hybridized to varying amounts of *Eco*RI-digested human placenta DNA (Figure 7, left). Clearly visible signals were obtained in as low as 1 μg total placenta DNA after overnight detection. To compare the sensitivity obtained with [^{32}P]-radioactively labeled probes the identical genomic blot was hybridized with the same, but [^{32}P]-radioactively labeled β-actin RNA probe (Figure 7, right). After overnight exposure to an X-ray film analogous sensitivity was obtained; thus, both methods are of equivalent sensitivity in the same detection period.

The digoxigenin-labeled β-actin-specific RNA probe was also used for identifying mature human β-actin mRNA (30) in total RNA from liver cells (Figure 8). Decreasing amounts of total RNA in the range between 1 and 0.01 μg were separated in denaturing gels and blotted to nylon membranes. The rather abundant mature and polyadenylated β-actin-mRNA was visible in as low as 0.1 μg of total RNA after overnight detection.

DISCUSSION

The digoxigenin hapten was utilized for non-radioactive labeling and detection of RNA probes. The labeling reaction was optimized with regard to high yield, high sensitivity and low

background. High yield was achieved by optimizing buffer composition as well as concentrations of NTPs and RNA polymerase. As the presence of DIG-UTP reduces the transcription rate, it was important to find a balanced ratio of DIG-UTP to UTP during the transcription reaction. The mixture of 35% DIG-UTP and 65% UTP is a compromise, which gives 1) a relatively high yield of labeled RNA, and 2) results in a distance of haptens within the digoxigenin-modified RNA which is suitable for obtaining high sensitivity and low background in hybridization reactions. As the nucleotide concentration is not limiting in the standard labeling reaction, up to 10 μg of full-length labeled transcripts can be synthesized which are up to 5 kb in length. The yield of 10 μg digoxigenin-labeled RNA is sufficient for 100 ml hybridization solution. The digoxigenin-labeled RNA probes are stable for several years and can therefore be prepared in advance, which ensures reproducible results.

Digoxigenin-labeled RNA probes can be used in all standard hybridization applications (2, 3, 8, 9, 26, 27, 39). The data presented in this publication show that in dot-, Southern- or Northern-blots as low as 0.1 pg homologous DNA or RNA can be detected within 16 hrs; human single-copy genes are detectable in 1 μg placenta DNA. The application of digoxigenin-labeled RNA probes for *in situ* hybridizations will be published elsewhere.

The digoxigenin hapten is linked with the spacer by an ether bond. Photodigoxigenin contains an alkali-stable spacer as well (25). Both types of nucleotide is stable against alkali-treatment; in contrast to analogous biotin nucleotides (11) digoxigenin-labeled probes can be size reduced by alkali-treatment, which is of advantage for some applications of *in situ* hybridizations (32, 33).

The optimal temperatures between 45 and 60°C for RNA:DNA-hybridizations were surprisingly high. However, these results were confirmed with different RNA probes. Important for reduction of background was the addition of an efficient blocking reagent during hybridization as well as in the first blocking step of the detection reaction. The formulation of a new modified blocking buffer with maleic acid enables the sterilization of five-fold concentrated blocking buffer, which is more convenient and efficient for inactivation of RNase activity.

An important feature of the detection of digoxigenin-labeled RNA probes is the use of an highly specific polyclonal sheep antibody also applied for the detection of digoxigenin-modified DNA (21–25). Removal of the Fc-portion of the antibody prior to conjugation with the alkaline phosphatase marker enzyme reduces unspecific binding and background.

Digoxigenin-labeled RNA probes have the same sensitivity as radioactively labeled probes (10) avoiding the handling of radioisotopes. Another advantage of digoxigenin-labeled RNA probes is the high resolution of closely spaced signals. This is due to the detection by an enzyme-catalyzed colour precipitation; precipitates are only formed exactly where digoxigenin-labeled hybrids are formed, the signal does not diffuse or spread. This feature is of special importance for *in situ* hybridizations.

In this publication we only described the detection of digoxigenin haptens by alkaline phosphatase and BCIP/NBT colour substrates. Alternative detection schemes are under evaluation including luminescent substrates like 1,2-dioxetanes (AMPPD) (40, 41) as well as alternative colour substrates [naphtol AS phosphate-diazonium salts (FAST dyes)] (42) for alkaline phosphatase, which both should increase the flexibility of application of digoxigenin-labeled RNA probes (Höltke,H.J., *et al.*, unpublished results). Furthermore, we have prepared alternative conjugates like anti-digoxigenin coupled to peroxidase (43) or fluorescent markers (fluorescein, rhodamine (44)); these kinds of alternative conjugates have already been applied for *in situ* hybridizations (45).

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